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Diagnostic Virology

W. Lawrence Drew, MD*

VIRUS CLASSIFICATION

All true viruses have a nucleic acid that is either DNA or RNA. Nucleic acid composition is the basis for a simple system of classifying viruses of clinical importance. The DNA viruses comprise six groups that cause human disease (Table 1); the RNA viruses may be divided into 11 such groups (Table 2).

ETIOLOGY OF VIRAL SYNDROMES

Certain specific syndromes may be caused by several different viruses. This is especially true of respiratory diseases, such as bronchiolitis, which is most commonly caused by either respiratory syncytial or parainfluenza viruses (Tables 3 and 4). Similarly, the spectrum of disease potential for any one virus type is quite broad. For example, influenza virus may cause illness ranging from upper respiratory infection to pneumonia. Table 5 illustrates similar considerations in several nonrespiratory diseases. Because several viruses may cause the same clinical syndrome, it is important to make a specific etiologic diagnosis. In this article I will review current techniques for viral diagnosis.

CYTOLOGY

The most readily available rapid technique is cytologic examination for the presence of characteristic viral inclusions. These intracellular structures may represent aggregates of virus within an infected cell or may be abnormal accumulations of cellular materials consequent to the viral-induced metabolic disruption. Papanicolaou smears may show these inclusions in single

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Table 1. *Viruses of Human Importance: DNA*

FAMILY	VIRAL MEMBERS
Pox	Variola, vaccinia, molluscum contagiosum
Herpesvirus	Herpes simplex, varicella-zoster, cytomegalovirus, Epstein-Barr virus
Adenovirus	Types one to eight most implicated in causing human disease
Papovavirus	Human papilloma (wart), progressive multifocal leukoencephalopathy agents (JC, SV40), BK
Hepatitis B	Hepatitis B
Parvovirus	Norwalk agent

cells or in large syncytial groups; Figure 1 shows these in a patient with herpes simplex infection of the cervix. Cytology is most commonly used to detect infections with herpes simplex virus (HSV) or cytomegalovirus (CMV), but is definitely less sensitive than culture.

DETECTION OF VIRAL ANTIGEN

Direct examination of virus-infected tissues or exudates may be performed with specific fluorescein-labeled viral antibody. This test can be completed within 1 hour and is a sensitive method for detecting the presence of viral antigen. However, the performance of the test requires considerable technologic expertise, and commercially available reagents are quite variable in quality. Fluorescent antibody identification has been used regularly for the diagnosis of infections with HSV, varicella-zoster (VZV), respiratory syncytial (RSV), and rabies viruses. We have found that VZV, which grows slowly or not at all in tissue culture, can be readily detected in smears from skin lesions by a fluorescent antibody method.⁶ In many laboratories direct FA is superior to culture for the diagnosis of VZV and RSV infections, because culture of these two viruses takes several days and may be falsely negative. Many viral antigens may also be detected by

Table 2. *Viruses of Human Importance: RNA*

FAMILY	VIRAL MEMBER
Orthomyxovirus	Influenza A, B, C
Paramyxovirus	Parainfluenza, mumps, measles, respiratory syncytial virus
Togavirus	Encephalitis (western equine, eastern equine, St. Louis, etc.), yellow fever, dengue, rubella
Bunyavirus	California encephalitis, Rift Valley fever
Coronavirus	Coronavirus
Reovirus	Types 1 to 3, rotavirus, Colorado tick fever
Picornavirus	Enterovirus: Polio, Coxsackie A, Coxsackie B, Echovirus, enterovirus 68-71, 72 (hepatitis A); Rhinovirus; Calicivirus, Norwalk virus
Arenavirus	Lymphocytic choriomeningitis, Lassa, Tacaribe (hemorrhagic fever agents)
Marburg, Ebola	
Rhabdovirus	Rabies
Retrovirus	Human T-cell leukemia, probable AIDS agent (HTLV-III/LAV)

Table 3. Most Common Etiologic Agents of Viral Respiratory Diseases in Infants and Children

SYNDROME	AGENTS
Upper respiratory tract infection	Rhinovirus, coronavirus, parainfluenza, adenovirus, respiratory syncytial virus, influenza
Pharyngitis	Adenovirus, coxsackie A, herpes simplex, EB virus, rhinovirus, parainfluenza, influenza
Croup	Parainfluenza, respiratory syncytial virus
Bronchitis	Parainfluenza, respiratory syncytial virus
Bronchiolitis	Respiratory syncytial virus, parainfluenza
Pneumonia	Respiratory syncytial virus, adenovirus, influenza, parainfluenza

indirect fluorescent antibody techniques. The recent development of in-vitro production of monoclonal antibodies should greatly enhance the specificity and sensitivity of IF as well as other immunodiagnostic methods in the future.

The following is a suggested approach to direct IF diagnosis.

Conjugates

Fluorescein isothiocyanate-conjugated antisera are reconstituted with phosphate-buffered saline (PBS), pH 7.4 to 8.0, aliquoted, and stored at -60°C until the time of use. These samples are then titrated at 1:5, 1:10, 1:20, and 1:40 dilutions in PBS to determine optimal working dilutions in infected cell cultures. (Optimal dilution is defined as that which gives 3+ to 4+ specific fluorescence in cells infected with homologous virus and no fluorescence in cells infected with heterologous virus or in uninfected cells.) This optimal dilution is then tested for cross-reactivity with heterologous viruses, as well as for nonspecific fluorescence in impression smears or frozen sections of normal, noninfected human tissues, such as brain and

Table 4. Most Common Etiologic Agents of Viral Respiratory Disease in Adults

SYNDROME	AGENTS
Upper respiratory tract infection	Rhinovirus, coronavirus, adenovirus, influenza, parainfluenza
Pneumonia	Influenza, adenovirus
Pleurodynia	Coxsackie B

Table 5. Etiologic Agents of Common Viral Syndromes

SYNDROME	AGENTS
Myocarditis	Coxsackie B
Pleurodynia	Coxsackie B
Herpangina	Coxsackie A
Febrile illness \pm rash	Echo, coxsackie
Infectious mononucleosis	EB virus, cytomegalovirus
Aseptic meningitis	Echo, coxsackie A & B
Encephalitis	Herpes simplex, togavirus, rabies, enteroviruses

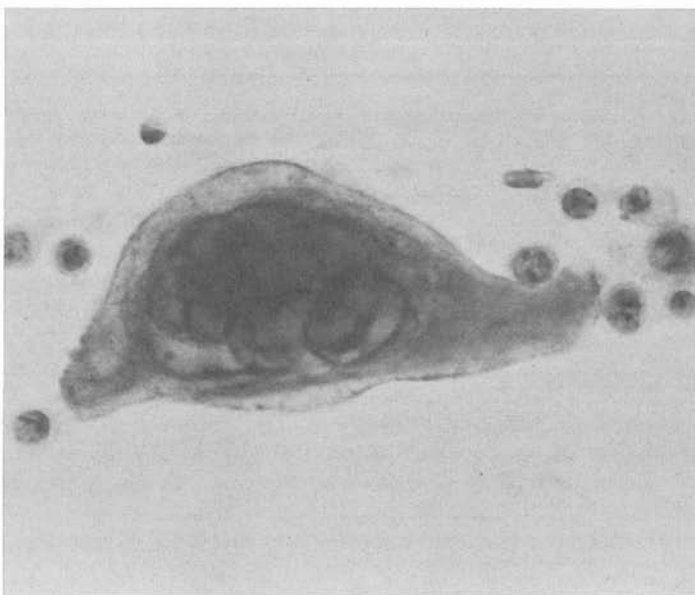


Figure 1. Papanicolaou smear showing multinucleated giant cell.

lung. In clinical use, appropriate controls include preimmune conjugates derived from the homologous animal source, conjugated antisera to heterologous viruses, and, when tissue sections or impression smears are used, normal, noninfected tissue controls. The conjugates are used on clinical specimens at a twofold lower dilution than for typing of tissue culture isolates.

Slides

Clean, plain, 1-mm-thick glass slides can be used for frozen sections or impression smears. For examination of resuspended cells, preprinted slides are preferred to slides prepared by spraying with Fluoroglide.

Specimens

Four types of specimens can be used for IF diagnosis: frozen sections, impression smears, lesion scrapings, and resuspended cells in centrifuged sediment.

Frozen sections should be cut to 3 to 4 μm in thickness, and lesion scrapings are best obtained with a scalpel blade and then smeared in spots on the slide. Swabs of lesions may be rolled onto the slide; rubbing should be avoided, because this can distort cell morphology.

Cell suspensions can usually be readily recovered from urine or other body fluids (for example, amniotic fluid by centrifugation). In addition, if appropriate vigor has been applied in the collection of swabs from the respiratory tract, conjunctiva, vagina, or skin lesions, the specimens in viral transport media can be utilized. These samples are blended thoroughly on a Vortex mixer, and the swabs are removed. The samples are then

centrifuged at 1200 to $1500 \times g$ for 30 minutes, and the supernatant is used for cell culture inoculation. The remaining sediment is suspended in just enough PBS (without phenol red) to yield a slightly turbid suspension, which is spotted on the wells of slides with a Pasteur pipette. The amount of PBS required for resuspension usually varies from 0.1 to 1.0 ml. All slides are labeled appropriately, allowed to air-dry completely, and then fixed in acetone at -20°C for 10 minutes. If slides must be transported from one laboratory to another, they may be sent air-dried at ambient temperature, or acetone-fixed at 4°C . Formalin-fixed tissues are *not* suitable for IF studies.

Staining

Appropriately diluted conjugate is overlaid on each specimen, and the slides are incubated at 35°C for 30 to 60 minutes in a humidified chamber. The slides are then washed with gentle agitation for two 5-minute periods in PBS. Cover slips (no. 1) are mounted with buffered glycerol (pH 8.0), and specimens are examined for fluorescence. A halogen-source microscope equipped with epifluorescence is ideal for this purpose, with observation at $600\times$ magnification. If oil immersion is used, low-fluorescence oil is necessary.

Interpretation

Strict criteria for interpreting fluorescence patterns must be applied. For example, nuclear and cytoplasmic staining are typical for influenza virus, adenoviruses, and the herpes viruses; cytoplasmic staining only is seen with RSV, parainfluenza, and mumps viruses; staining within multinucleated giant cells is typical of measles viruses. If the pattern of fluorescence is appropriate, and proper controls have been employed, the specificity of the test is high. In fact, for some agents such as VZV, direct IF is often considerably more sensitive than culture.

False-positive results can be obtained if the previously mentioned criteria are not strictly applied. Specimens that contain yeasts, mucus, or leukocytes can be especially misleading. Also, CSF sediment results should be viewed with great caution; leukocytes from any source may possess Fc receptors that can be responsible for nonspecific binding of conjugates. It is extremely important to attempt virus isolation on specimens submitted for fluorescent antibody staining to verify results, to enhance the overall sensitivity of diagnosis, and to properly standardize the conjugates. When both cultures and IF examination are performed, false-negative IF tests will be observed. In some instances, these false-negative tests result from specimens with very low virus titer, requiring many days for detection in culture. False-negative fluorescent assay results have occurred with critical specimens such as brain biopsies for HSV encephalitis; culture "back-up" is therefore mandatory.

Rapid Detection of Viral Antigen in Tissue Culture

In addition to direct detection of viral antigens by clinical specimens, immunofluorescent antibodies may also be used to rapidly detect the presence of a virus such as CMV in tissue culture. By centrifuging urine

onto tissue culture monolayers and staining at 24 to 36 hours after inoculation, this virus may be detected without waiting many days for cytopathogenic effect to develop.⁷

SOLID-PHASE IMMUNOASSAYS

Solid-phase immunoassays (SPIAs) for the detection of antigens or antibodies use, as an indicator, a radioactive label (for RIA) or the action of an enzyme label on a substrate (for ELISA). Several excellent reviews on these procedures and their application to viral diagnosis have been published.^{2, 4, 13, 15}

Applications

SPIA techniques have been successfully applied to the detection of hepatitis B, rotavirus, hepatitis A antibody, adenovirus, HSV, RSV, influenza A virus, CMV, and group A coxsackievirus. Commercial kits are available for the detection of rotaviruses as well as HB_sAg, HB_sAb, HB_cAb, and HAV IgM and IgG. Fluid specimens are most appropriate for detection of viral antigens by ELISA. Serum may be used directly in the test; urine and feces may need homogenization with saline and preliminary clarification by centrifugation to be suitable in the test. Nasopharyngeal or sputum specimens should be collected into appropriate transport media and diluted 1:5 to 20 per cent *n*-acetyl cysteine before use in the test. Radioimmunoassay has been used to detect hepatitis B and herpes simplex antigens in tissue culture. More recently, other immunologic techniques such as ELISA have been applied to the detection of these and other viral antigens both directly and in culture.

Advantages and Disadvantages

Enzyme immunoassays provide the advantage of relatively stable and nonradioactive reagents, and the results can be either qualitative or quantitative. ELISAs are usually more sensitive than all other assays except RIA. The equipment necessary for ELISAs can be quite simple and objective. The major drawback of all SPIAs is that the quality of specimens such as respiratory, genital, or skin swabs cannot be assessed. This is in marked contrast to IF. Specific disadvantages of ELISAs include the variability of solid-phase carriers and the carcinogenicity of certain chromogenic substrates. RIAs require expensive equipment and also involve exposure to carcinogens. In addition, disposing of these hazardous materials poses considerable difficulty. Most SPIAs also require a day or overnight incubation with several washing procedures. For the optimal use of SPIAs in rapid viral diagnosis, several specimens should be examined for antigen in a given run. Thus, these techniques are best suited for diseases such as viral gastroenteritis, viral hepatitis, or respiratory infections, in which many specimens are being submitted to the laboratory at a given time. Although a single specimen could be analyzed, quite often an alternative assay (IF, EM, and so on) might be more appropriate.

ELECTRON MICROSCOPY

Several reviews of the procedures and utility of electron microscopy (EM) in diagnostic virology have been published.^{1, 3, 5, 10} The procedure is surprisingly simple for an experienced electron microscopist and can be completed within 15 minutes of receipt of the specimen. While it would be inappropriate to purchase an electron microscope solely for a diagnostic virology laboratory, an existing electron microscope in an institution could be used. EM techniques are most applicable for the investigation of viral infections in which the titer of virus in specimens is at least 10^6 to 10^7 particles per ml. EM is most commonly utilized for feces examination, because rotaviruses as well as Norwalk agents, adenoviruses, coronaviruses, and caliciviruses may be seen and identified as the cause of the illness. The Norwalk agent virus, an important cause of gastroenteritis, is not easily visible by standard EM in feces and usually requires enhancement by immune EM to permit detection. Immune EM allows visualization of virus particles that may be present in numbers too small for easy direct detection. The addition of specific antiserum to the test suspension causes the virus particles to form antibody-bound aggregates, which are more easily detected by EM than are single virus particles. Other specimens such as vesicle fluid, brain tissue, solid warty tissue, urine, or serum can be negatively stained with minimum preparation to yield positive results. If concentrations of viruses are lower in these specimens or others, techniques to enhance visualization are necessary. Enhancement techniques may include pseudo-replication, agar gel diffusion, ultracentrifugation, or immune EM.

Diseases for which EM can play a role in rapid diagnosis include gastroenteritis, herpes simplex infections, varicella-zoster (especially in the immunocompromised patient), congenital CMV infection, hepatitis B, molluscum contagiosum, and warts.

In most instances, viruses seen by EM can be detected by alternative methods. For example, HSV and VZV viral antigens can be detected by IF techniques, and rotavirus in stool specimens can be detected by latex agglutination, ELISA, or counterimmunoelectro-osmophoresis. Nonetheless, EM can be quite useful in a diagnostic virology laboratory, especially in the examination of feces from patients with gastroenteritis, because—in addition to rotaviruses—Norwalk virus, adenoviruses, coronaviruses, and caliciviruses may be seen and identified as the cause of the illness.

EM can also be used to detect herpesvirus particles in a brain biopsy from a patient with encephalitis, and CMV particles may be seen in high concentration in the urine of congenitally infected infants but may require some form of enhancement for EM detection. Although hepatitis B viral particles can be seen in serum with EM, very sensitive and convenient methods for viral antigen detection such as RIA or ELISA are usually employed. Human papilloma virus (common warts), Orf virus, and molluscum contagiosum poxvirus can be viewed in homogenates of solid biopsy tissue, but examination of these specimens is rarely indicated.

Finally, EM can be used to hasten the identification of a virus that has been recovered in tissue culture and to detect viral and mycoplasma contaminants in cell cultures.

Laboratory Procedures for Electron Microscopy

Specimen Collection and Preparation. Specimens should be collected as described subsequently and transported at ambient temperature in sealed containers to prevent drying.

Fluid Specimens. Depending upon the type of clinical specimen, centrifugation may help to provide a better preparation. Feces can be prepared by making a 10 to 20 per cent suspension in distilled water and then clarifying with a bench centrifuge if necessary.

Vesicle fluid should be collected from unbroken lesions by using a tuberculin syringe containing a small amount of distilled water. A sheath should be placed over the needle and tape should be applied to the plunger and sheath before the syringe is transported to the laboratory inside an appropriate container.

Urine that is cloudy should be clarified by centrifugation at $2500 \times g$ for 30 minutes. The supernatant fluid will usually yield virus more readily if as large a volume as possible—for example, 10 to 20 ml—is centrifuged for 1 hour at $15,000 \times g$ and then the resuspended pellet is stained. Techniques of enhancement (mentioned subsequently) have proven useful for urine specimens.

Serum contains many low-molecular-weight proteins that need to be washed out by diluting with an equal volume of distilled water. As with urine, centrifugation for 1 hour at $15,000 \times g$ is necessary, and the serum may even require recentrifugation to provide a clean specimen.

Tissue Specimens. Biopsy or autopsy tissue is cut into small (1-mm) cubes and placed on a metal planchet for freezing and thawing (five cycles). An alternative method involves mechanical homogenization using a mortar and pestle or homogenizer. After this, a relatively smooth suspension can be achieved by mixing the homogenate in small amounts of distilled water by using a fine-bore Pasteur pipette. Best results are achieved by differential centrifugation and staining of the supernatant fluid with phosphotungstic acid.

Other Specimens. Other, less commonly examined specimens may be submitted for EM examination. Conjunctival scrapings usually contain small amounts of tissue. CSF seldom yields a virus. Both of these specimens should be processed directly without centrifugation. Sputum specimens should be diluted in saline and then homogenized or treated with 20 per cent *n*-acetyl cysteine. Differential centrifugation or some form of enhancement is usually necessary to concentrate the specimen.

Staining. The most appropriate laboratory technique for the examination of clinical specimens is negative staining. The most commonly used stain is phosphotungstic acid as a 2 to 4 per cent solution adjusted to a pH of 6 to 8 with 1 N potassium hydroxide and stored as a working solution at 4°C. Formvar-carbon-coated, 400-mesh copper grids provide the most versatile carriers for specimen viewing. The technique involves placing a drop of sterile distilled water on a waxed surface. A grid held by fine forceps is touched to the drop, held upright, and stained with a drop of phosphotungstic acid. Excess fluid is removed with a torn strip of filter

paper, and the specimen is dried before examining by EM. The total process takes about 5 minutes.

Techniques to Enhance Virus Visualization. Other techniques have been devised to enhance the sensitivity of EM for detecting viruses. These include pseudoreplication and agar gel diffusion for concentrating rotaviruses in feces and CMV in urine. Ultracentrifugation and immune EM have also been employed for concentrating rubella virus, Norwalk agent, and hepatitis A virus.

SEROLOGY

Up to about a decade ago, serology was utilized as a primary procedure in diagnosing viral infections. More recently, this approach has been replaced in many cases by methods designed to detect the viral agents or their antigens as quickly as possible. However, several situations still exist in which serologic diagnosis can be extremely helpful.

Interpretation

Acute-Phase Serum. When a virologic work-up is planned for an individual patient, it is generally useful to obtain at least 2 to 3 ml of acute-phase serum and store it in the freezer. This may become valuable, particularly if virus detection subsequently fails or the interpretation of an isolate is equivocal. In these instances a convalescent-phase serum may be requested 2 to 3 weeks after the first, and appropriate selection of antigens to be tested can be made.

Single Sera. In some cases, a single serum may be studied and appropriately interpreted. Virus-specific immunoglobulin M (IgM) antibody usually rises during the first 2 to 3 weeks of infection and persists for several weeks to months, eventually becoming replaced by IgG antibody. Thus, an elevated titer of specific IgM antibody suggests a recent primary infection by the virus in question, and this may be further supported by demonstrating a fall in IgM antibody in follow-up sera. Detection of specific IgM has been used with success in the diagnosis of infections due to VZV, Epstein-Barr virus (EBV), and CMV, and rubella and coxsackieviruses and is currently the procedure of choice to establish a recent or active infection due to hepatitis A. The methods to be employed in IgM-specific antibody determination vary considerably, including sucrose density gradient centrifugation, column chromatography, staphylococcal protein A absorption, and solid-phase immunoassays such as IF assay, ELISA, and RIA. These methods vary in sensitivity, convenience, and applicability. Several limitations of interpretation must be kept in mind. It is now recognized that IgM-specific antibody responses are not always restricted to primary infections; reactivation or reinfection may result in IgM responses, particularly in CMV, HSV, EBV, and VZV infections. In addition, patients may continue to produce IgM-specific antibody to rubella or CMV for many months after a primary infection. Heterotypic IgM responses can also occur—for example, antibody responses to CMV in EBV infections and vice versa. Other pitfalls include falsely low or negative IgM titers due to competition by

IgG antibody for antigen-binding site and false-positive reactions due to rheumatoid factor; both these errors appear to be most common in solid-phase assays employing IF.

Both false-negative and false-positive IgM fluorescent antibody results have been frequently encountered in screening sera from newborns for specific antibody to CMV and rubella virus.¹² In some but not all cases, false-positive results can be attributed to rheumatoid factor of maternal origin. False-negative results might occur as a result of competition between high levels of maternal IgG and low levels of fetal IgM in cord area.

Other uses of single sera include screening infant blood for certain antibodies of the IgG class, known popularly as the TORCH screen. Antibody to *Toxoplasma gondii* (TO), rubella (R), CMV (C), and HSV (H) is measured in an effort to determine possible congenital infection with these agents; however, the utility of these tests has been misunderstood. They are more useful in excluding a possible infection than in proving an etiology. If, for example, rubella antibody is absent, then the infant almost certainly does not have congenital rubella infection. To diagnose active rubella infection in such a baby, viral cultures and additional serologic studies are required. Other uses of single sera include CMV antibody screening to eliminate the transmission of CMV antibody-positive blood to seronegative babies or other immunocompromised patients and rubella antibody screening to identify women requiring vaccination.

If there is adequate knowledge of the specific levels of antibody titers achieved in individuals during acute infection and afterward, the height of a titer in a single serum sample may aid in a presumptive diagnosis of recent infection. For example, a hemagglutination-inhibition antibody titer of 1:160 or greater to western equine encephalitis or St. Louis encephalitis virus or a complement fixation titer of 1:128 or greater to influenza B virus would be supportive of a recent experience with the agent. However, it is important to remember that such interpretive criteria cannot be applied to all infections, because titers to many viruses can persist at varying levels. Thus, titers in single sera must be interpreted cautiously in most cases.

False-Positive Serologic Results. The majority of serologic interpretation is based upon conversion from seronegativity to positivity or a fourfold or greater rise in antibody titer between paired sera. However, such apparently significant antibody titer rises may not always be significant. An example of this is the occasional rise of antibody to CMV in patients with influenza A or *Mycoplasma pneumoniae* infections, suggesting stress reactivation of CMV by the latter agents. False-positive serologic responses may also result from cross-reactions to related antigens; for example, an antibody rise to parainfluenza virus may actually result from infection with mumps virus. Also, anamnestic antibody responses may falsely suggest seroconversion or cause an apparent fourfold or greater rise in antibody titer.

False-Negative Serologic Results. Infants may not develop complement fixing and the antibody responses to RSV, influenza virus, and other agents. They may also fail to develop IgM antibody responses despite congenital infection with CMV or rubella virus. Severely immunocompromised pa-

tients may fail to develop antibodies, although this is comparatively infrequent.

Serologic Panels

Selection of antigens for testing with paired sera in cases in which a virus is suspected but not detected can sometimes be made on the basis of clinical syndrome, the known local epidemiology of particular viruses, and the age of the patient. This has led to the concept of serologic "batteries" or "panels."

Central Nervous System Syndromes. HSV, mumps, western equine encephalitis, eastern equine encephalitis, St. Louis encephalitis, and California encephalitis viruses and perhaps lymphocytic choriomeningitis virus and EBV may be included in a battery of tests for a CNS syndrome. Although herpes simplex antigen is included in the panel, a rise in antibody titer is not sufficient to diagnose HSV encephalitis. A large share of viral CNS illness, especially aseptic meningitis, is caused by the enterovirus group, but the multiplicity of serotypes and the cumbersome serologic methods necessary for their diagnosis usually make it impractical to include them in a battery. When one or two enteroviruses have been shown to be epidemic in an area one summer it is possible to pick up some additional cases by carrying out neutralization tests on paired sera employing only the enterovirus or enteroviruses in question.

Respiratory Syndromes. Depending upon the age of the patient, the antigen panel for testing respiratory syndromes might include influenza A and B, RSV, parainfluenza 1, 2, and 3, adenoviruses, *M. pneumoniae*, and chlamydia. For example, RSV and parainfluenza might be routinely tested in infants and young children but not in adults.

Exanthems. To test exanthems, an antigen battery would include measles and rubella. If the disease is vesicular, HSV and VZV should be included.

Myocarditis and Pericarditis. Antigens from group B coxsackievirus, types 1 through 5, and perhaps influenza A and B viruses (depending upon epidemiologic circumstances) would make up the battery tested for myocarditis and pericarditis. Although there are numerous viruses that have been implicated in inflammatory diseases of the heart and its covering membranes, the group B coxsackieviruses, types 1 through 5, have been considered to account for nearly 50 per cent of the cases. Unfortunately, much of the clinical illness is expressed at a time when standard methods of virus detection are likely to fail, and serologic diagnosis must be attempted.

Special Considerations

The full interpretation of serologic results may require testing for the presence of antibody to several different antigenic components of a single virus; two notable examples are EBV and hepatitis B virus.

In those instances of EBV infections that cannot be diagnosed by the usual clinical criteria and heterophile antibody tests, specific IF antibody tests can be performed (Table 6). These include (1) IgG antibody to viral capsid antigen (VCA), which appears early in infection and usually persists

Table 6. *Epstein-Barr Virus Serology*

SITUATION	IgG-VCA	IgM-VCA	EA	EBNA
No past infection	—	—	—	—
Acute infection	+	+	+	—
Convalescent phase	+	+ or —	+ or —	+
Past infection	+	—	—	+
? Chronic or reactivation	+	—	+	+

for life; (2) antibody to early antigen, which appears in most patients and persists only during the active phase of infection (weeks to months); and (3) antibody to EBV nuclear antigen (EBNA), detected by anticomplement IF, which appears 2 to 4 weeks after onset and usually persists for life. In the early phase of acute infection, IgM-specific VCA titers are usually elevated; however, reactivation of infection may also provoke a similar response. Presence of *both* VCA and EBNA antibody in an acute or convalescent-phase serum suggests a past infection. Recent EBV infection is suggested by any of the following: (1) IgM antibody to VCA, (2) presence of VCA antibody and absence of EBNA, (3) rising titer of EBNA, or (4) presence of elevated VCA and early antigen antibodies.

Acute hepatitis B virus infection is primarily diagnosed by detection of the surface antigen (HB_sAg) in serum. Antibody to HB_sAg (anti-HB_s) appears weeks to months after the virus has been eliminated and is a marker of past infection with relative immunity to reinfection. Antibody to the core antigen (anti-HB_c) appears during infection and persists for variable periods thereafter. In some patients with acute hepatitis B, HB_sAg has declined to an undetectable titer and the anti-HB_c may be the only marker of acute hepatitis B infection. When this occurs, and the anti-HB_s has not yet developed, the patient is said to be in the "core window." Because anti-HB_c may persist for a long time, its presence is not diagnostic of acute hepatitis B. The single best test for this purpose is the 1 gm antibody to core antigen, the 1 gm anti HB_c. Another component of hepatitis B virus known as e antigen may also be sought in HB_sAg-positive sera, as well as its corresponding antibody. The presence of hepatitis B e antigen is correlated with infectivity, and its antibody is considered to be a marker for a lower risk of transmission. These latter antigen tests are important in epidemiologic studies, but are rarely necessary to perform for clinical diagnostic purposes.

VIRAL CULTURE

The "gold standard" test for proving the etiology of a viral syndrome is recovery of the agent in tissue culture. This procedure is surprisingly simple to perform.

Just as multiple media are used in bacteriology, several different types of tissue cultures are inoculated with each viral specimen. Detection of viral presence in the cultures is accomplished by observing the cytopathogenic effect (CPE), a change in the appearance of the tissue culture cells.

With experience, a technologist can distinguish the CPE characteristics of certain virus groups and make a presumptive judgment of which virus has been isolated. This judgment is aided by noting which of the several tissue cultures exhibit CPE, and how rapidly and extensively CPE had developed. In many clinical situations, this presumptive identification suffices for final identification.

Although more definitive biologic or immunologic procedures may be performed, just as in bacteriology they may be unnecessary. Flat, lactose-fermenting colonies of indole-positive bacteria recovered from a urine specimen may be reasonably presumed to be *Escherichia coli*. Similarly, an agent recovered from a genital lesion that produces a herpes-like CPE in several types of tissue culture within 1 or 2 days may be reasonably presumed to be HSV.

Viral cultures are routinely incubated for 10 days unless CMV is suspected. Blind subculture of specimens negative for CPE is unnecessary in the clinical laboratory; the yield is slight, and such a low titer of virus may reflect a carrier state rather than a pathogenic relationship. Respiratory tract cultures without CPE must be hemadsorbed to detect certain viruses (for example, parainfluenza and mumps) that may not develop CPE. In this procedure, a suspension of guinea pig red blood cells is added to the tubes, and the tissue culture monolayers are examined for adherence of these cells. If adherence occurs, a hemadsorbing virus may be present.

Clinical laboratories need not maintain stocks of embryonated eggs or colonies of suckling mice. It is true that certain viruses (such as certain serotypes of coxsackie A virus, and togaviruses, formerly arboviruses) will not be isolated without them, but they are few and not worth the greatly increased effort. If the clinical illness warrants, specimens to be cultured using these systems may be sent to reference laboratories. Other common viruses that are not isolated by the standard method described include rubella, measles, and rotaviruses. Specimens from patients suspected of infection with these agents also should be sent to reference laboratories for special culture procedures or, in the case of rotaviruses, for direct electron microscopy or antigen detection.

In contrast to the intrinsic delay of serologic studies, the results of viral culture can be surprisingly rapid. The first 1000 viruses isolated in our laboratory required an average of 4.1 days for detection, and most isolates of herpes simplex and influenza A viruses were reported within 2 to 3 days of receipt of the specimen. Herrmann⁹ has reported that 44 per cent of all viral isolates could be reported within 4 days of specimen receipt. In addition to the two "rapid growers" already mentioned, he was able to report more than two thirds of all enterovirus isolates to the physician within 5 days of obtaining the specimen.

CMV and RSV have been regarded as "slow growers" in the past. At the Mayo Clinic, only 57 per cent of the RSV isolates were recovered within 10 days of culture.⁹ Recently, Hall and Douglas⁸ have improved RSV recovery by directly inoculating cultures at the patient's bedside with nasal wash specimens. Using this procedure and Hep-2 tissue cultures, they were able to detect two thirds of the RSV isolates within 4 days of culture. The bedside inoculation seems to be the key step, because we

have achieved similar results by inoculating a simple nasopharyngeal or throat swab directly into the tissue cultures. Direct bedside inoculation of throat cultures in our hospital also has improved the recovery of CMV, from only 43 per cent recovery at 10 days to 80 per cent in 8 days. By direct inoculation of urine into tissue culture, we have occasionally detected CMV CPE within 1 day.

Specimen Requirements for Viral Culture

Source. Selection of appropriate specimens is complicated, because several different viruses may cause the same clinical disease. For example, several different types of specimens may be submitted from patients with CNS disease: CSF (enterovirus, mumps virus, and perhaps HSV type 2); throat (enterovirus); and stool or rectal swab (enterovirus). In addition, blood should be collected as an acute-phase specimen in case subsequent serologic tests are necessary (mumps, virus, HSV, arbovirus). Many considerations, however, allow the clinician to select the most appropriate specimens. For example, during the summer, when enteroviral meningitis is prevalent, throat and stool specimens should certainly be submitted, in addition to CSF. On the other hand, the development of encephalitis in children after the acquisition of several mosquito bites in wooded areas endemic for California encephalitis virus suggests that a blood specimen for antibody testing would be optimal. Further examples include CNS disease following parotitis (urine and CSF specimens for mumps virus) or a focal encephalitis with a temporal lobe localization preceded by headaches and disorientation (brain biopsy for HSV). On the other hand, in respiratory syndromes it is generally only necessary to submit a throat/nasopharyngeal specimen; stool and urine isolates would be irrelevant. Other considerations are as follows.

Throat, Nasopharyngeal Swab, Aspirate. For recovering viruses, nasopharyngeal aspirates are superior to swabs, but the latter are considerably more convenient. Throat swabs are probably adequate for recovering entero- and adenoviruses and HSV, whereas nasopharyngeal specimens are definitely superior for recovering RSV and are probably better for parainfluenza viruses. Nasal specimens are optimal for recovering rhinoviruses.

Rectal Swabs and Stool Specimens. Most cases of viral gastroenteritis are now known to be due to viruses that cannot be cultivated in cell cultures. Fecal specimens from such cases may be examined for nonculturable agents, such as rotavirus, by electron microscopy or by antigen detection (see subsequent discussion). Stool cultures are useful in patients suspected to have enterovirus disease—for example, aseptic meningitis, myopericarditis, and hand, foot, mouth disease. Enteroviruses in the feces of such patients support but do not definitely prove that these agents are causing the illness. Available evidence indicates that stool specimens are more productive than rectal swabs. Generally, infectivity of viruses that are surrounded by a lipid membrane (such as HSV and CMV) are destroyed by gastric acidity, and therefore they will not be excreted in active form in the feces.

Urine. CMV, mumps, and adenovirus are the viruses most frequently recovered from urine by the laboratory. In cases of CNS disease due to

mumps, the virus can be isolated from urine when specimens from other sites are negative. The recovery of CMV from urine is increased two- to threefold by processing several specimens. Urine specimens submitted for culture of CMV can be inoculated directly into cell cultures. Alternatively, they may be centrifuged, and either the supernatant urine or the sediment, resuspended in a small volume, can be used as inoculum. Recent studies indicate that centrifuging the urine onto the tissue culture monolayer is the best of all these procedures.⁷

Generally, examination of the urine sediment for cytomegalic inclusion-bearing cells is not a sensitive method for diagnosing CMV infections. Viral isolation is at least fourfold more sensitive in both infants and adults.

Dermal Lesions. Fluid and cells from vesicles are superior to specimens prepared from ulcers or crusts for both culture and direct stains.

CSF, "Sterile" Fluids (pleural, peritoneal, pericardial, joint). These fluids should be inoculated as quickly as possible into tissue culture.

Eye. A nasopharyngeal swab may be used to obtain secretions from the palpebral conjunctiva. Eye scrapings should be obtained by an ophthalmologist or other trained person.

Blood. CMV viremia may be present in symptomatic infection and has been correlated with lethal pneumonia in bone marrow transplant recipients. Leukocytes, in which this and other viruses reside, may be collected more efficiently by a Ficoll-Hypaque-Macrodex technique than by the conventional "buffy-coat" method. Anticoagulated blood or a clot can be used for isolation of arboviruses, and serum is suitable for recovering enteroviruses.

Tissue. Tissue explants and cells grown in cell cultures after dispersal of the cells from tissue fragments have provided higher rates of viral isolation than homogenized specimens. Presumably, viral inhibitors may be released into the homogenate, resulting in lower recovery rates in the disrupted cells. Lung (CMV, influenza virus, adenovirus) and brain (HSV) are the most productive tissue sources for viral isolates. As with urine sediment, the cytologic detection of CMV inclusions in tissue is at least three to six times less sensitive than viral isolation.

Timing of Specimen Collection. Proper timing of specimen collection is essential for adequate recovery of viruses. Specimens should be collected early in the acute phase of infection. Studies with respiratory viruses indicate that the mean duration of viral shedding may be 3 to 7 days. Also, HSV and VZV may not be recovered from lesions beyond 5 days after onset. Isolation of an enterovirus from the CSF may only be productive within 2 to 3 days after onset of the CNS manifestations.

Transport of Specimens to the Laboratory. The shorter the interval between collection of a specimen and its delivery to the laboratory, the greater the potential for isolating an agent. When feasible, inoculate all specimens other than blood, feces, and tissue into culture tubes at the patient's bedside. These are then transported to the laboratory promptly. Any material may be used for swabs; however, calcium alginate may inactivate HSV. In general, the following statements hold, but there may be a few exceptions: (1) Never leave a specimen at room or incubator temperature; (2) When it is impossible to deliver a specimen immediately,

it should be refrigerated and packed in shaved ice for delivery to the laboratory within 12 hours of collection.

Several types of media have been used for the transport of viral specimens. Most of these have been selected for this purpose based either on tradition or on data obtained by testing the survival of laboratory strains of virus. A recent double-blind prospective study to compare the recovery of viruses from the upper respiratory tracts or dermal lesions of children, using three types of transport media, showed no statistically significant differences between modified Stuart, modified Hanks, or Liebovitz-Emory media.¹¹ It is generally believed that protein (serum, albumin, gelatin) incorporated into a transport medium enhances the survival of viruses in transit; however, two studies have indicated that HSV survives as well in Stuart or Hanks protein-free medium.¹⁴ The "Culturette" (Modified Stuart's Bacterial Transport Medium, Marion Laboratories Inc., Kansas City, MO) appears to be satisfactory for short-term (up to 4 hours) transport.

Improper storage can significantly reduce viral culture yields. After a freeze-thaw cycle, significant losses in infectivity titer occur with lipid-envelope viruses (HSV, CMV), but not with agents such as adeno- and enteroviruses, which have a coat of protein only. For example, a laboratory strain of HSV held for 1 to 3 days at -20°C and then thawed had reductions in infectious titer of 100-fold or more. In contrast, when stored for 1 to 3 days at 4°C in Hanks or broth medium, there was no loss of infectivity in two thirds of the specimens.¹⁴ In another study, only 3 of 45 (7 per cent) strains of CMV failed to produce cytopathic effects after storage for 7 days at 4°C .¹² Storage at room temperature was unsatisfactory. Alternatively, once a specimen is received into the laboratory, cryoprotectants such as sorbitol may be added, but even then viral suspensions must be frozen and thawed under rigid conditions (-70°C when freezing, 25°C per minute when thawing) to achieve maximum recovery of the virus. *Thus, for short-term (less than 5 days) transit or storage, specimens for viral culture should be held at 4°C rather than frozen.*

Cell Culture Systems

Primary Monkey Kidney (PMK). This is an excellent system for recovery of myxoviruses and many enteroviruses, and also may support growth of adenoviruses, RSVs, and measles viruses. These cells are occasionally contaminated by simian viruses (most commonly SV 5 and SV 40), a problem minimized by the addition of antisera to these agents in the cell culture media.

Human Fetal Diploid (HFD). An example is foreskin fibroblasts (Fig. 2). These fibroblastic cells have the dual advantages of being relatively inexpensive and susceptible to a broad spectrum of viruses. They are useful in the isolation of VZV, HSV, adenovirus, picornavirus, and RSV, and are the only cells in which CMV is recovered.

Hep-2 Continuous Cell Line. These epithelial cells, derived from a human cancer, are an excellent cell system for recovering adenovirus, HSV, and especially RSV.

The availability of commercial cell cultures and media greatly facilitates the work involved in virus isolation. If purchased commercially, upon

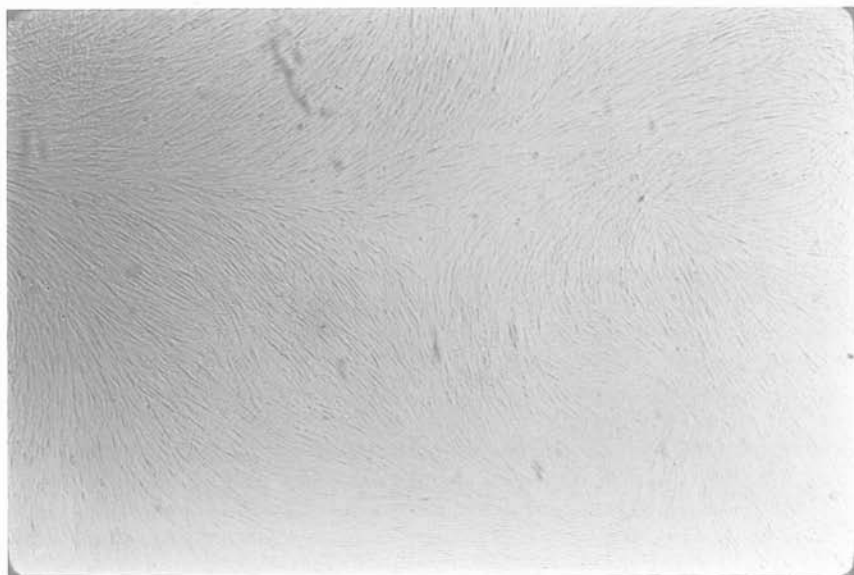


Figure 2. Normal diploid fibroblast tissue culture cells.

arrival in the laboratory, tubes are examined microscopically. If an adequate monolayer of cells is present, the growth medium is replaced by 2 to 3 ml of fresh maintenance medium prior to storage at 35°C. If the monolayer is incomplete, the growth medium is replaced by 2 to 3 ml of fresh growth medium. HFD and Hep-2 cultures can be prepared in a clinical laboratory rather than purchased commercially.

The majority of clinically significant viruses can be recovered in the cell systems described previously. Specimens submitted for the isolation of viruses requiring other types of cell cultures (for example, coxsackie A, togaviruses) are forwarded to reference laboratories. Some laboratories prefer additional cell cultures such as RD (rhabdomyosarcoma) or BGM (continuous monkey kidney) for recovering coxsackie A and B viruses, respectively.

Interpretation of Viral Culture Results

The interpretation of virologic data must be based on knowledge of the normal viral flora in the site sampled, the clinical findings, and the epidemiologic behavior of viruses. In some situations, serologic studies may be necessary to support or refute the possible association of a virus isolate with a disease.

Viruses in Tissue and Body Fluids. In general, the detection of any virus in host tissues, CSF, blood, or vesicular fluid can be considered highly significant. Recovery of viruses other than CMV in urine may be diagnostic of significant infection—for example, mumps virus and adenovirus type 11, associated with acute hemorrhagic cystitis. However, recovery of CMV from urine can also be difficult to interpret. The CMV isolate may

merely reflect active, asymptomatic virus replication or may indicate a significant infection in the patient. Viruria in the first 3 weeks of life establishes a diagnosis of congenital CMV infection, whereas the onset of viral excretion after 4 weeks of life reflects intrapartum or postpartum infection. Diagnosis of acquired CMV infections in older patients usually results from a combination of findings, including positive cultures from any site, illness known to be compatible with CMV, reasonable exclusion of other potential etiologic agents, and support by specific serologic or histologic data or both.

Virus Isolation from Other Sites. Upper respiratory tract (or specimens obtained by traversing the upper airways), vaginal, and fecal cultures vary greatly in terms of their significance. At one extreme, isolates such as measles, mumps, influenza, parainfluenza, and RSV are very significant, because asymptomatic carriage and prolonged shedding of these viruses are unusual. Conversely, other viruses can be shed without symptoms and for periods ranging from several weeks (enteroviruses) to many months (adenoviruses, HSV, CMV). Examples include HSV and CMV in the oropharynx and vagina, adenoviruses in the oropharynx and intestinal tract and enteroviruses in the intestinal tract. VZV, HSV, CMV, adenoviruses, and EBV may remain latent for long periods and then become reactivated in response to a variety of stressful stimuli, including other infectious agents. In this setting, their detection may have no significance (VZV is an exception) or may merely represent a secondary problem complicating the primary infection (for example, HSV "cold sores" in patients with bacterial sepsis).

Adenovirus isolates are common in infants and young children. Based on the epidemiology and observed serological responses, it has been found that, in febrile and respiratory syndromes, simultaneous isolation of these agents from both the throat and feces has greater probability of association with that illness; isolation from the throat, but not the feces, has a lesser probability of association, and isolation from the feces has the least diagnostic significance. In these latter situations, interpretation of the significance of the culture results can be aided by adenovirus serologic studies.

Enteroviruses are also most commonly found in infants and children, particularly during the late summer and early autumn seasons. A knowledge of the relative frequency of virus shedding among various age groups in a particular locale is extremely helpful in assessing significance of results of throat or stool cultures; for example, the peak prevalence of enteroviruses in the stools of toddlers during the late summer may range from greater than 20 per cent in subtropical climates to 5 per cent in temperate zones. Even in the latter areas, carriage rates may approach 30 per cent in infants during periods of enterovirus activity. Shedding of enteroviruses in the throat is relatively transient (usually 1 to 2 weeks), whereas fecal shedding may last 4 to 16 weeks. Thus, in a clinically compatible illness, isolation of an enterovirus from the throat supports a stronger temporal relationship to the disease than does an isolate from only the feces. When echovirus or coxsackievirus is recovered from the stool only, it may be helpful to study the patient's serum for an antibody rise against that particular viral isolate.

HSV is unusual in a fecal culture; in such cases, it usually represents either severe disseminated infection or infection of the anus or perianal

areas. If this virus is obtained from an anal or rectal culture of a sexually active patient with signs of nervous system disease, such as aseptic meningitis or lumbosacral myeloradiculitis, it may indicate the etiology. Detection of HSV in the upper respiratory tract may have no meaning other than nonspecific stress reactivation unless typical vesicles or ulcers are also seen. Because of the stress-related phenomenon, isolation of HSV in the throat or mucocutaneous lesions of patients with encephalitis cannot be interpreted as causative of the CNS disease. Currently, the definitive way to establish a diagnosis of herpes simplex encephalitis is by direct demonstration of the virus in a brain biopsy. In neonates, however, isolation of the virus from any site should raise the possibility of potentially severe infection.

Isolation of adenoviruses, HSV, VZV, and some enteroviruses from the cornea and conjunctiva in cases of inflammatory disease at these sites usually establishes the etiology of the infection.

Significance of Negative Virologic Results

There are numerous occasions when the laboratory is asked to perform studies with the intent of ruling out specific viral agents. The ability to exclude the presence of a virus depends greatly on the sensitivity of the detection systems being used. The most important prerequisites include (1) proper communications with the laboratory *before* initiating studies; (2) procurement of the appropriate specimens in the *early* phase of acute illnesses; (3) careful attention to the procedure of collection, transportation, and processing of specimens; and (4) avoidance of fungal or bacterial contamination of processed cultures. When all these criteria are fulfilled, it is usually possible to interpret negative data with a high degree of confidence.

REFERENCES

1. Almeida JD: Practical aspects of diagnostic electron microscopy. *Yale J Biol Med* 53:5-18, 1980
2. Bidwell DE, Bartlett A, Vollar A: Enzyme immunoassays for viral diseases. *J Infect Dis* 136(Suppl):S274-S278, 1977
3. Chernesky MA: The role of electron microscopy in diagnostic virology. In Lennette D, Specter S, Thompson K (eds): *Diagnosis of Viral Infections: The Role of the Clinical Laboratory*. Baltimore, University Park Press, 1979, pp. 125-142
4. Daugharty H, Ziegler DW: Radioimmunoassay in viral diagnosis. In Kurstak E, Kurstak C (eds): *Comparative Diagnosis of Viral Diseases. Volume II*, New York, Academic Press, 1977, pp. 459-488
5. Doane FW, Anderson N: Electron and immune electron microscopic procedures for diagnosis of viral infections. In Kurstak E, Kurstak C (eds): *Comparative Diagnosis of Viral Diseases. Volume II*, New York, Academic Press, 1977, pp. 505-539
6. Drew WL, Mintz L: Rapid diagnosis of varicella-zoster virus infection by direct immunofluorescence. *Am J Clin Pathol* 73:699-701, 1980
7. Gleaves CA, Smith TF, Shuster EA, Pearson GR: Rapid detection of cytomegalovirus in MRC-5 cells inoculated with urine specimens by using low-speed centrifugation and monoclonal antibody to an early antigen. *J Clin Microbiol* 19:917, 1984
8. Hall CB, Douglas GR Jr: Clinically useful method for the isolation of respiratory syncytial virus. *Infect Dis* 131:1-5, 1975

9. Herrmann EC Jr: Efforts toward a more useful viral diagnostic laboratory. *Am J Clin Pathol* 56:681-686, 1971
10. Hsiung GD, Fong CKY, August MJ: The use of electron microscopy for diagnosis of virus infections: An overview. *Progr Med Virol* 25:133-159, 1979
11. Huntoon CJ, House RF Jr, Smith TF: Recovery of viruses from three transport media incorporated into culturettes. *Arch Pathol Lab Med* 105:436, 1981
12. Stagno S, Pass RF, Reynolds DW, et al: Comparative study of diagnostic procedures for congenital cytomegalovirus infection. *Pediatrics* 65:251, 1980
13. Voller A, Bartlett A, Bidwell DE: Enzyme immunoassays with special references to ELISA techniques. *J Clin Pathol* 31:507-520, 1978
14. Yeager AS, Morris JE, Prober CG: Storage and transport of cultures for herpes simplex virus, type 2. *Am J Clin Pathol* 72:977, 1979
15. Yolken R: Enzyme-linked immunosorbent assay (ELISA): A practical tool for rapid diagnosis of viruses and other infectious agents. *Yale J Biol Med* 53:85-92, 1980

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